Title: The NO-donating pravastatin derivative (NCX 6550) reduces Splenocyte Adhesion and ROS Generation in Normal and Atherosclerotic Mice

Dever G, Spickett CM, Kennedy S, Rush C, Tennant G, Monopoli A & Wainwright CL

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland UK (GD, CMS, SK, CR, GT). School of Pharmacy, The Robert Gordon University, Aberdeen, Scotland UK (CLW). Nicox Research Institute, Milan, Italy (AM)

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Running title: NO-releasing pravastatin and splenocyte adhesion

Author for Correspondence

Professor Cherry L Wainwright

School of Pharmacy

The Robert Gordon University

Schoolhill

Aberdeen AB10 1FR

Scotland UK

Tel: 01224 262450

Fax: 01224 262555

E-mail: c.wainwright@rgu.ac.uk

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Abbreviations:

APES - 3-aminopropyl triethoxysilane; ApoE-/- - Apolipoprotein E receptor knockout; CL – chemiluminescence; ICAM-1 – inter-cellular adhesion molecule 1; MCP-1 – monocyte chemoattractant protein 1; MPO – myeloperoxidase; NADPH oxidase – nicotinamide adenine dinucleotide phosphate-H; NCX 6550 – Nitrated pravastatin ((1S-[1α(βS*,δS*),2α,6α,8β-(R*),8aα]]-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-1-naphtaleneheptanoic acid 4-(nitrooxy)butyl ester); NO – nitric oxide; NOX - NOX-based NAD(P)H oxidases; PAR-1 – protease activated receptor 1; PHOX – phagocyte oxidase; PMA – phorbol myristate acetate; ROS – reactive oxygen species; SOD – superoxide dismutase; VCAM-1 – vascular cell adhesion molecule 1

Abstract

Statins possess anti-inflammatory effects that may contribute to their ability to slow atherogenesis while nitric oxide (NO) also influences inflammatory cell adhesion. This study aimed to determine whether a novel NO-donating pravastatin derivative, NCX 6550, has greater anti-inflammatory properties compared to pravastatin in normal and atherosclerotic ApoE -/- mice. C57Bl/6 and ApoE -/- mice were administered pravastatin (40 mg/kg), NCX 6550 (48.5 mg/kg) or vehicle orally for 5 days. *Ex vivo* studies assessed splenocyte adhesion to arterial segments and splenocyte ROS generation. NCX 6550 significantly reduced splenocyte adhesion to artery segments in both C57Bl/6 (8.8 ± 1.9 vs 16.6 ± 6.7 % adhesion; $P<0.05$) and ApoE -/- mice (9.3 ± 2.9 vs 23.4 ± 4.6 % adhesion; $P<0.05$) concomitant with an inhibition of endothelial ICAM-1 expression. NCX 6550 also significantly reduced PMA-induced ROS production that was enhanced in isolated ApoE -/- splenocytes. Conversely, pravastatin had no significant effects on adhesion in normal or ApoE -/- mice, but reduced the enhanced ROS production from ApoE -/- splenocytes. In separate groups of ApoE -/- mice, NCX 6550 significantly enhanced endothelium-dependent relaxation to carbachol in aortic segments precontracted with phenylephrine ($-\log EC_{50}$ 6.37 ± 0.37) compared to both vehicle ($-\log EC_{50}$ 5.81 ± 0.15; $P<0.001$) and pravastatin ($-\log EC_{50}$ 5.57 ± 0.45; $P<0.05$) treated mice. NCX 6550 also significantly reduced plasma MCP-1 levels (648.8 pg/ml) compared to both vehicle (1191.1 pg/ml; $P<0.001$) and pravastatin (847±71.0 pg/ml; $P<0.05$) treatment. These data show that NCX 6550 exerts superior anti-inflammatory actions compared to pravastatin, possibly through NO-related mechanisms.

Introduction

Atherosclerosis is now generally acknowledged to be an inflammatory disease where inflammation develops at certain predilection sites in response to endothelial injury. Attachment of leukocytes to atherosclerotic blood vessels (Ramos et al., 1999), coupled with upregulation of the vascular adhesion molecules VCAM-1 and ICAM-1 (Nakashima et al., 1998) is a fundamental step in the development of atherosclerosis. Recently, adhesion of cultured murine monocytoid WEH1 78/24 cells to artery segments of ApoE -/- mice has been demonstrated (Li et al., 2005) supporting the notion that a hyper-inflammatory state exists in developing atherosclerosis. This has been attributed to increased levels of a number of cytokines, which act to elevate adhesion molecule expression. Thrombin, in addition to playing a role in the coagulation cascade, is also involved in the regulation of inflammation and has been shown to induce monocyte adhesion to endothelial cells (HUVEC) through induction of ICAM-1 and increased expression of VCAM-1, P-selectin and E-selectin (Kaplanski et al., 1998) due to an action at PAR-1. Consequently, thrombin has been implicated in atherogenesis (Coughlin, 2005). However, there has been no direct demonstration of a hyper-inflammatory response to thrombin in atherosclerotic blood vessels. The first aim of the current study was therefore to compare adhesion of splenocyte preparations, commonly used as a source of immune cells, to thrombin-challenged arterial tissue from normal (C57Bl/6) and atherosclerotic (ApoE -/-) mice.

The value of introducing the lipid-lowering statins into the management of patients with coronary artery disease has been illustrated through the significant benefit of these drugs in primary (Shepherd et al., 1995) and secondary prevention of symptomatic coronary heart disease (Scandinavian Simvastatin Survival Study (4S), 1994). Detailed analyses of data from these trials, however, suggest that lipid-lowering by statins does not solely account for the significant clinical outcomes, and that statins possess additional (pleiotropic) effects beyond their lipid-lowering capacity (Downs et al., 1998). Among the reported pleiotropic effects of statins, demonstrations of their anti-inflammatory and anti-adhesive effects are abundant (Fischetti et al., 2004; Sparrow et al., 2001; Stalker et al., 2001). Furthermore fluvastatin (Bandoh et al, 2003), and other statins, have been

shown to inhibit formation of reactive oxygen species (ROS) by inflammatory cells. However, while these effects are readily demonstrated following acute challenge with supratherapeutic concentrations *in vitro*, the effects *in vivo* often require prolonged administration before they are observed. Although nitric oxide (NO) was originally identified as a key mediator in the maintenance of vascular tone it also exerts anti-inflammatory effects. NO, either generated endogenously or released from NO-donating molecules, inhibits leukocyte adhesion through a reduction in endothelial expression of adhesion molecules such as P-selectin (Davenpeck et al., 1994) and ICAM-1 (Berendji-Grun et al., 2001). Recent studies have reported superior anti-inflammatory properties of novel NO–releasing statins (nitrostatins), over the respective native statins in RAW 264.7 murine macrophage cells (Ongini et al., 2004; Rossiello et al., 2005). The NO-donating moiety of nitrostatins is similar to other nitro compounds such as nitro aspirin, which yields NO through metabolic hydrolysis resulting in relatively long lasting plasma levels of NO (Muscara et al, 2001). Furthermore, studies conducted with the NO-releasing derivative of pravastatin, NCX 6550 ((1S-[1α(βS*,δS*),2α,6α,8β-(R*),8aα]]-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-1-naphtalene -heptanoic acid 4-(nitrooxy)butyl ester)), showed that the compound given to hypercholesterolemic CD1 mice is equally effective as equivalent doses of the native statin at lowering cholesterol (Monopoli et al, Personal Communication). Thus, NO released by these molecules may provide a more rapid anti-inflammatory action than can be achieved with a native statin, while still affording a reduction in cholesterol levels. Thus the aim of the present study was to compare the effects of short-term (5 days) *in vivo* administration of NCX 6550 and native pravastatin on *ex vivo* splenocyte adhesion to arterial segments, splenocyte ROS production, and endothelial ICAM-1 expression in tissues from normal (C57Bl/6) and atherosclerotic (ApoE -/-) mice. In addition we determined the effects of these interventions on endothelium-dependent vasorelaxant function and plasma MCP-1 levels in ApoE -/- mice.

While many studies that investigated leukocyte-endothelial adhesion have used *in vitro* cell models, such as myeloid cell adhesion to HUVEC monolayers (McGettrick et al, 2006), we chose to use a more physiological model involving *in vivo* dosing with statins

followed by *ex vivo* measurement of isolated splenocyte adhesion to arterial tissue. Although this approach has the limitation of being a static model of vascular adhesion, it has the advantage of allowing atherosclerosis-susceptible arteries to be studied, in contrast to intravital microscopy, which is a dynamic model that allows the detection of adhesion in the presence of shear stress, but which involves visualising microvascular beds that are not generally susceptible to atherosclerotic plaque development, such as the mesenteric bed.

Methods

Materials

C57Bl/6 (Harlan, UK Ltd) and ApoE -/- (Charles River Laboratories, France) mice were bred in house at the University of Strathclyde. NCX 6550 (1S-[1α(βS*,δS*),2α,6α,8β-(R*),8aα]]-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-1-naphtaleneheptanoic acid 4-(nitrooxy)butyl ester) was synthesised at NicOx (Bresso, Milan, Italy). All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Experimental Design

Forty C57Bl/6 (18-24 g) and fifty two ApoE -/- (26-35 g) age matched mice of either sex were employed in the study, under a project licence issued under the UK Animals (Scientific Procedures) Act 1986. The ApoE -/- mice were fed on an atherogenic diet (1% cholesterol, 5% lard) for 12 weeks post-weaning, and control normocholesterolaemic C57Bl/6 mice were fed normal laboratory chow. The mice were then employed for the following studies:

(1) A group of C57Bl/6 (n=10) and ApoE -/- (n=10) mice were used in preliminary experiments to determine any difference between the strains with respect to the adhesive response to thrombin, ICAM-1 expression and splenocyte ROS generation.

(2) Three groups (n=10 per group) of mice of each strain were administered either vehicle (DMSO: Castor oil: PEG 400: Water, 1: 2: 7: 10 v/v/v/v), native pravastatin (40 mg/kg) or NCX 6550 (equimolar dose, 48.5 mg/kg) by oral gavage every day (at 10 a.m.) for 5 days. One hour after the final dose the mice were euthanised by CO_2 asphyxiation. Heparinised blood was obtained by cardiac puncture immediately following euthanasia for subsequent measurement of plasma cholesterol levels using standard assay kits (R-Biopharm).

(3) Three groups (n=4 per group) of ApoE -/- were administered either vehicle, pravastatin or NCX6550 (all as above) for 5 days. Immediately following euthanasia blood was collected by cardiac puncture into heparinised tubes for measurement of plasma MCP-1 using an ELISA assay kit (Insight Bioscience, UK) and the aorta harvested for determination of blood vessel function.

Splenocyte Isolation and Radiolabelling

Splenocyte suspensions were prepared by disrupting the spleens over a 200 μm mesh (Cadisch Ltd) into 3 mL of RPMI-1640 medium (Dutch Modification: Invitrogen Ltd, UK) containing 10% foetal calf serum (Invitrogen). The resulting cell suspensions were passed through a 200 μm mesh and centrifuged at 125 *g* for 10 min. The supernatants were removed, the pellets containing the cells agitated with 4 mL of distilled H_2O for 30 seconds (to lyse erythrocytes), followed by addition of 4 mL 1.8% NaCl to restore isotonicity. The splenocyte suspensions were filtered through a 200μm mesh, centrifuged and the resulting cell pellets re-suspended in 2 mL of RPMI. Cell density was determined using a haemocytometer, and the suspension diluted as necessary to achieve a final density of $1x10^6$ cells Ll^{-1}. 1ml of the re-suspended leukocytes was labelled for 1hr at 37°C in a humidified chamber with 185 kBq of ^{51}Cr (Amersham Biosciences, UK); the cells were agitated every 15 min to minimise cell sedimentation. The cells were washed twice with RPMI and re-suspended in RPMI to $1x10^6$ cells mL^{-1}.

Splenocyte Characterisation

Splenocyte preparations were characterised in samples from three separate C57Bl/6 and three ApoE -/- mice using flow cytometry. Briefly, cell suspensions were incubated with FcR blocking buffer (anti-CD 16/32 hybridoma supernatant, 10% mouse serum and 0.1% azide) for 5 min to prevent binding of antibody to cells via Fc regions. The cell suspensions were then incubated with a mixture of cell lineage specific antibodies for 40 min at 4°C. B lymphocytes were identified using flourescein isothiocynate (FITC) conjugated anti-CD45R/B220 (clone RA3-6B2; Pharmingen, Oxford, UK), $CD4^+$ T lymphocytes using Peridinin chlorophyll protein-cyanin 5.5 (PerCP) conjugated anti-CD4 (clone GK1.5; BD Pharmingen, Oxford, UK) and myeloid cells using Phycoerythrin (PE) conjugated anti-CD11b (clone M1/70; BD Pharmingen, Oxford, UK). The cells were washed in FACS buffer (PBS, 2% FCS and 0.1% azide) prior to acquisition using a BD FACSCanto flow cytometer with FACSDiVa software (BD, UK). FlowJo software (Tree Star Inc, USA) was used for three colour analysis. To ascertain which cell types were adhering to the artery surface, cells were added to pinned out segments of aorta for 30

minutes and adherent cells were harvested by addition of ice cold PBS solution. The suspensions of adherent cells were incubated with the same three antibodies and run through the flow cytometer.

Assessment of Splenocyte Adhesion to Aortic Segments

The method employed was a modification of a method utilising rabbit tissue developed in our laboratory to determine the effects of vascular injury on inflammatory cell adhesion (Kennedy et al., 2000). Homologous aortic lengths were removed and cut into two segments (aortic arch and thoracic aorta), which were pinned out luminal-side up onto Sylgard blocks (Dow Corning, Germany). The artery segments were incubated with 10µl of 10 U mL^{-1} thrombin for 10 min in a humidified chamber (37°C), washed and then incubated with a 5 µL aliquot of the labelled leukocytes for a further 30 min. The segments were then washed with RPMI, transferred into microtubes and assayed for ^{51}Cr in a gamma counter (Cobra™ Auto-gamma®, Packard, Canberra Company, UK). Aliquots (5 µL) of labelled and unlabelled cells were also counted to allow calculation of leukocyte adhesion using the following equation:

(1) % Adhesion = $(\gamma_{artery} - background)/(\gamma_{splenocytes} - background) \times 100$

where γ_{artery} = count from the artery and $\gamma_{splenocytes}$ = count from a 5 µL aliquot of labelled splenocytes.

Measurement of Splenocyte ROS Generation

A 450 µL aliquot of unlabelled splenocyte suspension ($1x10^6$ cells mL^{-1}) was diluted 1:1 with phosphate-buffered saline (PBS) in a plastic cuvette containing a stir bar, which was then placed in the pre-warming chamber of a chemiluminometer (Chronolog Corporation) for 2 min. 100 µL of 400 µg mL^{-1} luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were added prior to the cuvette being transferred to the photomultiplier compartment. After 1 min the cells were stimulated with 5-100 ng mL^{-1} (final concentrations) of phorbol myristate acetate (PMA), and a cumulative chemiluminescent signal was measured for 15 min.

Immunocytochemistry

Upon completion of the adhesion assay, artery segments were fixed in formalin solution (4% formaldehyde) for 48 h and stored in PBS at 4°C. The tissues were subsequently processed and embedded in blocks of paraffin wax. 4 μm transverse sections were cut and mounted on APES-treated slides and oven-dried for 1hr at 60°C. Sections were then stained for ICAM-1 with goat anti-human/anti-mouse ICAM-1 as the primary polyclonal antibody (1:50; R&D Systems, UK) and biotinylated rabbit anti-goat secondary antibody (1:400; Dako, USA) using the streptavidin-horse radish peroxidase (Vector, UK) method as described previously in detail (Kennedy et al., 2000). The sections were then counterstained with haematoxylin prior to mounting in DPX (VWR International). Sections were subsequently subjected to semi-quantitative scoring analysis by one of us (CW) blinded to the treatment. Sections were scored from 0-3, where 0= none/background; 0.5= focal staining of the endothelium; 1= mild circumferential positivity on the endothelium; 2= moderate circumferential positivity on the endothelium; 3= intense circumferential positivity on the endothelium.

Assessment of endothelial function

Endothelial function was measured using a small artery wire myograph (Danish Myo Technology, Aarhus, Denmark). Briefly, 2mm segments of thoracic aorta were mounted on two 40μm stainless steel wires and normalised using a previously published method. Arteries were bathed in Krebs' solution, maintained at 37ºC and aerated with 95% O_2 and 5% CO_2 throughout. Following 30 mins of equilibration, all arteries were constricted by addition of a previously determined EC_{50} of phenylephrine (0.2 μM). Once contraction had stabilised, endothelium-dependent relaxation was measured by cumulative addition of carbachol (10^{-9} M–10^{-4} M). Maximum relaxation (E_{max}) and -logEC_{50} for each group of mice was calculated by fitting sigmoidal curves to each complete data set using GraphPad Prism version 4 (GraphPad Software Inc.).

Measurement of Plasma MCP-1 Levels

Plasma MCP-1 levels were measured using a mouse CCL2 ELISA kit (Insight Bioscience, Wembley, UK). Briefly, NUNC Maxisorp flat-bottom, high protein binding capacity 96-well plates were coated overnight at 4°C with 100 μL / well purified anti-mouse MCP-1 capture antibody (1:250 in coating buffer [Insight Bioscience]), after which they were aspirated and washed 3 times with 300 μL / well wash buffer (0.05% Tween-20 in PBS). Non-specific binding was blocked by incubation with 200 μL / well assay diluent (10% foetal bovine serum in PBS) for 1 hour at room temperature, the wells washed and standards (0-2000 pg mL^{-1} recombinant mouse MCP-1) and plasma samples (100 μL / well) added. The plate was then incubated at room temperature for 2 hours, aspirated and washed and 100 μL / well detection antibody (1:250 in assay buffer) added followed by incubation at room temperature for 1 hour. After a further aspiration and wash, 100 μL of Avidin-HRP (1:250 in assay buffer) was added to each well and the plate was incubated at room temperature for 30 min, after which each plate was subjected to seven cycles of aspirate and wash. 100 μL of 60 μg mL^{-1} TMB (tetramethylbenzidine) substrate solution was added to each well; the plate incubated at room temperature for 15 min, and the reaction stopped by addition of 50 μL / well of stop solution (1 M H_3PO_4). The plate was read on a SpectraMax 190 plate reader (Molecular Devices Corporation, Sunnyvale, California): MCP-1 levels were determined by subtracting the absorbance values at 570 nm from those at 450 nm and extrapolating values from the standard curve.

Statistics

Values shown are mean ± s.e.m. Multiple group comparisons for % splenocyte adhesion, ROS generation, plasma cholesterol and MCP-1 levels and immunostaining scores were performed using One-way ANOVA and Dunnett's Post-Hoc test. For the functional studies, complete dose response curves were compared by Two-Way ANOVA, while E_{max} and $-\log EC_{50}$ values were compared by One-way ANOVA and Tukey post-hoc test.

Results

Characterisation of splenocyte preparations

Splenocytes isolated from the C57Bl/6 mice all contained similar proportions of $CD4^+$ T lymphocytes, B-220^+ B lymphocytes and $CD11b^+$ myeloid cells. Together these accounted for approximately 80% of the cell population. Analysis of the cell types adhering after 30 minutes contact with arterial segments revealed that all three cell types were present in approximately the same proportion as was found in the splenocyte isolates (Figure 1). In ApoE-/- mice both the isolated splenocytes and the adherent cells exhibited a greater proportion of B lymphocytes than in the C57Bl/6 mice, although the proportion of $CD4^+$ T lymphocytes and $CD11b^+$ myeloid cells were similar in both strians (Figure 1). The remaining 10-20% of cells in both strains were most likely $CD8^+$ and $\gamma\delta^+$ T lymphocytes.

Influence of atherosclerosis on adhesive response to thrombin

In a preliminary series of experiments performed in a separate group of mice that did not receive any form of oral drug administration, a significant difference was observed in the adhesive response to thrombin in both the aortic arch and the thoracic aorta from ApoE -/- mice compared to normal mice (Figure 2A). Furthermore, splenocytes isolated from ApoE -/- mice generated substantially more ROS on challenge with PMA than splenocytes from C57Bl/6 mice (Figure 2B).

Thrombin-stimulated splenocyte adhesion and ICAM-1 expression

Figure 3A illustrates splenocyte adhesion to arterial segments from vehicle- and drug-treated C57Bl/6 mice following five days of pre-treatment. Native pravastatin had no effect, whereas NCX 6550 caused a reduction in splenocyte adhesion which, in the aortic and thoracic segments, was statistically significant compared to both the vehicle and pravastatin groups ($P<0.05$). NCX 6550 also inhibited adhesion in the thoracic segment in the ApoE -/- mice, whereas pravastatin did not (Figure 3B); no significant reduction in adhesion to the aortic segment with NCX6550 was observed, presumably due to the greater variability within this group ($P=0.17$) . There was no significant difference in the extent of ICAM-1 expression in thrombin-treated artery segments from vehicle controls

for either strain of mouse (Figure 4). NCX 6550 significantly reduced the extent of ICAM-1 staining observed in the segments from C57Bl/6 and ApoE -/- groups compared to vehicle-treated mice. Pravastatin, on the other hand, only reduced ICAM-1 expression in thrombin-treated segments from C57Bl/6 mice and not the ApoE -/- mice.

Splenocyte Reactive Oxygen Species Generation

Treatment with pravastatin had no effect on the extent of ROS generation from C57Bl/6 splenocytes, whereas NCX 6550 significantly reduced ROS generation in response to the highest concentration of PMA tested (Figure 5A). In contrast, both pravastatin and NCX 6550 significantly attenuated the enhanced ROS production in the ApoE -/- splenocytes to a similar degree (Figure 5B).

Endothelial Function

Treatment with NCX 6550 significantly enhanced endothelium-dependent relaxation in response to carbachol (Figure 6A) in ApoE -/- mice by causing a significant shift to the left of the dose response curve (EC_{50} 0.43 ± 0.37 μM compared to 1.55 ± 0.15 μM in vehicle treated mice; $P<0.05$) and an increase in E_{max} (67.55 ± 9.4% relaxation) compared to vehicle treated mice (E_{max} 39.1 ± 3.6%; $P<0.05$). Pravastatin did not cause any shift in the dose response curve (EC_{50} 2.73 ± 0.45 μM), but the maximum relaxant response was increased (E_{max} 63.4 ± 16.9%; $P<0.05$ compared to vehicle).

Plasma MCP-1

Both pravastatin and NCX 6550 reduced circulating MCP-1 levels (847.2 ± 71.0 $pgmL^{-1}$, $P<0.01$ and 648.8 ± 47.4 $pgmL^{-1}$, $P<0.001$ respectively) compared to vehicle control (1191.1 ± 176.1 $pgmL^{-1}$). MCP-1 levels in NCX 6550 treated mice were significantly ($P<0.05$) lower than levels measured in mice treated with pravastatin.

Plasma Cholesterol Levels

Plasma cholesterol levels in vehicle–treated ApoE -/- mice (1835 ± 345 μg mL^{-1}) were significantly higher ($P<0.01$) than in vehicle-treated C57Bl/6 mice (550 ± 83μg mL^{-1}). Plasma cholesterol levels were not affected by 5 day treatment with either pravastatin

(428 ± 153 and 2526 ± 350 μg mL^{-1} in C57Bl/6 and ApoE -/-, respectively) or NCX 6550 (305 ± 47 and 1628 ± 392 μg mL^{-1} in C57Bl/6 and ApoE -/-, respectively).

Discussion

Hypercholesterolaemia increases thrombin-stimulated splenocyte adhesion and ROS generation

In the present study we have shown that *in vitro* thrombin-induced adhesion of splenocytes to autologous arterial segments is enhanced in ApoE -/- mice compared to wild type controls. Although there was a higher proportion of B lymphocytes in the splenocyte preparations from ApoE -/- mice (51.4 ± 0.5% of the total cell population) compared to C57Bl/6 mice (33.6 ± 4.2%), our finding that for each strain the adherent cell populations were made up of similar ratios of these three cell types implies that the increased adhesion is not due to the increased B lymphocyte adhesion alone. Regarding the molecular basis of this enhanced adhesion, ICAM-1 is known to play a key role in firm adhesion of monocytes and lymphocytes in response to thrombin (Nie et al., 1997). However, our immunocytochemical determination of ICAM-1 expression does not support the notion that increased ICAM-1 expression is responsible for the increased adhesion in ApoE -/- arteries, although ROS-induced increases in binding affinity of ICAM-1 (Sellak et al., 1994) may play a role. Alternatively, we have found that expression of the thrombin receptor PAR-1 is increased in arteries from ApoE -/- mice (Pugh et al., unpublished), which might explain the enhanced response to thrombin.

Blood vessels from atherosclerotic animals (Stokes et al., 2002) and humans with coronary artery disease (Spiekermann et al., 2003) generate elevated levels of ROS. Our observation that splenocytes from ApoE -/- mice similarly generate substantially greater amounts of ROS is, to our knowledge, the first demonstration of this phenomenon, although the relative contribution of each cell type in the splenocyte suspensions to the total CL signal is unknown. Elevated ROS generation from leukocytes has been described in obese (Dandona et al., 2001) and Type II diabetic (Orie et al., 2000) patients, but data from patients with atherosclerosis is conflicting (Eid et al., 2002; Araujo et al., 1995). ROS generation by monocytes and neutrophils in disease models is well established, but evidence is now mounting that T-lymphocytes also generate ROS (Williams & Kwon, 2004), for example by the lipoxygenase pathway (Los et al., 1995), mitochondrial electron transport chain (Griendling et al., 2000) or NOXs (Lambeth,

2004), although there is no information regarding the effect of hypercholesterolaemia on these systems. The present use of luminol as a chemiluminescent probe measures total ROS production by the splenocyte preparations, which precludes determining whether the enhanced CL signal from ApoE -/- splenocytes is due to overproduction of superoxide or other ROS species. We have found previously (Lim et al., 2006) that a combination of SOD, catalase and sodium azide cannot completely inhibit CL generated by mouse splenocytes, implying that superoxide, hydrogen peroxide and myeloperoxidase products do not account for all the ROS generated and that other ROS (such as hydroxyl radical, singlet oxygen, lipid peroxide and nitric oxide) may also contribute to the CL signal.

The NO-donating pravastatin derivative (NCX 6550), but not pravastatin, inhibits thrombin-induced splenocyte adhesion in ApoE -/- mice

The key finding of this study was that NCX 6550 significantly reduced splenocyte adhesion to arterial tissue from both normocholesterolaemic and hypercholesterolaemic mice and inhibited ICAM-1 expression in arterial segments challenged with thrombin. In contrast, native pravastatin did not attenuate thrombin-stimulated adhesion, although it did reduce the expression of ICAM-1 in C57Bl/6, but not in ApoE -/- mice. This apparent dissociation between an anti-adhesive response and inhibition of ICAM-1 expression supports the findings in untreated animals. The failure of pravastatin to inhibit splenocyte adhesion *ex vivo* deviates from the growing body of evidence that statins, including pravastatin, exert some of their effects through anti-inflammatory mechanisms that are unrelated to lipid lowering (Schonbeck & Libby, 2004). However, most studies that demonstrate an anti-inflammatory effect of statins employed treatment periods longer than the 5-day period in the present study, suggesting that long-term treatment is required to observe an anti-inflammatory effect. For example, 2 and 4 weeks treatment with rosuvastatin was required to attenuate adhesion of a monocyte cell line to aortic segments and vascular ROS production in ApoE -/- mice (Li et al., 2005). Interestingly, in that study cholesterol levels were reduced by rosuvastatin, whereas in the present study no observable effects of either pravastatin or NCX 6550 were detected (presumably due to the different duration of drug treatment). Moreover, most *in vitro*

studies demonstrating an anti-inflammatory effect of statins have employed concentrations in the micromolar range, whereas *in vivo* data suggest that plasma concentrations in the nanomolar range may have an effect on leukocyte trafficking and recruitment. This has led to the notion that cell adhesion and migration *in vivo*, which is a dynamic environment, may be more sensitive to inhibition by statins than adhesion under static *in vitro* conditions. Although in the present study pravastatin was administered *in vivo*, the adhesion measurements were performed *ex vivo* under static conditions, which could explain the lack of effect of pravastatin.

The superior ability of NCX 6550 over native pravastatin, to reduce splenocyte adhesion *ex vivo*, to improve endothelial function and to reduce MCP-1 levels suggests that it is the NO moiety on this compound that is responsible for its anti-adhesive effect. Using spectroscopy to measure nitrosylhaemoglobin in rat whole blood, Ongini et al. (2004) have demonstrated a linear, time-dependent increase in NO release from NCX 6550 that was consistent with slow NO release kinetics. The quantity of NO released was suggested to parallel that produced by eNOS under physiological conditions. The increase in local NO levels resulting from NCX 6550 treatment could result in an anti-inflammatory effect since NO is known to interfere with the release of a number of inflammatory mediators (such as caveolin-1 and NFκB) and the expression of adhesion molecules (Guzik et al., 2003). An alternative explanation for the superior effect of NCX 6550, however, could be attributed to a physicochemical, rather than a pharmacological difference between the two compounds, since pravastatin is known to be weakly effective *in vitro* because of its low lipophilicity, while the different physicochemical properties of NCX 6550 make it more lipophilic and consequently increase its penetration into cells (Ongini et al., 2004).

Both NCX6550 and pravastatin inhibit splenocyte ROS production

In contrast to the findings with splenocyte adhesion and ICAM-1 expression, both pravastatin and NCX 6550 attenuated the increase in ROS generation from ApoE -/- splenocytes. As would be expected after such a short period of administration (5 days) neither drug reduced plasma cholesterol and therefore this effect is unlikely to be

mediated through a lipid lowering effect. However, this shared property does imply that it is an effect mediated through the statin molecule. It has been demonstrated that statins can reduce ROS generation from vascular tissue by reducing levels of p22phox mRNA (Wassmann et al., 2001) and can act directly as ROS scavengers (Bandoh et al., 2003). The present novel finding that statins can also inhibit ROS generation from inflammatory cells that are intimately involved in atherogenesis and plaque rupture offers a further mechanism by which these compounds exert their highly beneficial effect. Interestingly, NCX 6550 also reduced ROS generation in response to the highest concentration of PMA tested in splenocytes from normocholesterolaemic mice. This action may be related to the NO moiety, since we have previously shown that NO-donating drugs can scavenge ROS generated by inflammatory cells (Demiryurek et al., 1997).

NCX 6550 and pravastatin on endothelial function

Among the proposed pleiotropic effects of statins is an improvement in endothelial function through an increased bioavailability of nitric oxide, a reduction in oxidative stress and the promotion of re-endothelialization (Wolfrum et al., 2003). Our present studies support this notion through the demonstration that pravastatin increased the maximum response to carbachol. However, under the same conditions, treatment with NCX 6550 exerted a superior effect on endothelial function by increasing the sensitivity to an endothelium–dependent vasodilator (as demonstrated by the left-ward shift in EC_{50}) as well as increasing maximum response. This is consistent with previous observations that NCX 6550 improves endothelial function in hypertensive rats (Presotto et al., 2005), and is likely due to the enhanced NO availability provided by the molecule.

NCX 6550 and pravastatin on MCP-1 levels

MCP-1 is known to mediate monocyte recruitment into vessel walls at sites of atherosclerosis and raised MCP-1 levels have been associated with cardiovascular disease risk factors. The reduction in MCP-1 levels seen here with pravastatin is consistent with numerous studies demonstrating the ability of statins to reduce MCP-1 (reviewed in Ballantyne and Nambi, 2005). As with the effect on endothelial function, NCX 6550 had a greater influence on MCP-1 levels than native pravastatin. The ability of NO to

modulate MCP-1 expression in endothelial cells is well documented (Zeiher et al., 1995) and it is therefore reasonable to propose that the enhanced activity of NCX 6550 is due to the presence of the NO-donating moiety.

Conclusions

Taken together, our findings suggest that NCX 6550, which retains the properties of the parent statin compound while having the added asset of slow nitric oxide release, is significantly more effective than native pravastatin in relation to several inflammatory markers. Clinically this may be important in terms of maintaining plaque stability and endothelial function that is compromised by raised circulating cholesterol levels, and the provision of these beneficial effects early on in drug treatment (i.e. within days rather than months) certainly warrants further investigation.

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Figure Legends

Figure 1: Percentage of T-lymphocytes, B-lymphocytes and myeloid cells present in mouse spleen cell isolates (panel A) and in populations of cells adhering to aortic segments following 30 mins incubation (panel B). Cell type was determined by flow cytometry using 3 colour analysis in cell preparations from C57Bl/6 and ApoE -/- mice, either directly using the splenocyte preparation or after adherence to artery segments and subsequent release using ice-cold PBS. $CD4^+$ T lymphocytes were identified using PerCP-conjugated anti-CD4; B lymphocytes were identified using FITC-conjugated anti-CD45R/B220; myeloid cells were identified by Phycoerythrin-conjugated anti-CD11b. The results are shown as mean ± s.e.m. (n=3). *P<0.05 compared to C57Bl/6 in both panels.

Figure 2: Thrombin-stimulated splenocyte adhesion to aortic and thoracic arterial segments from ApoE -/- and C57Bl/6 mice (panel A). Arterial segments were treated with thrombin for 10 mins prior to incubation with ^{51}Cr-labelled splenocytes for 30 mins at 37^{o}C. Adhesion is expressed as a percentage of the total cells applied to the artery segment and results correspond to mean ± s.e.m. (n=10 for both groups). The generation of ROS from isolated splenocytes in response to PMA was measured using luminol-dependent chemiluminescence (panel B). Results are shown as mean ± s.e.m. (n = 6 for wild type controls and 10 for ApoE -/-). *P<0.05 compared to C57Bl/6 in both panels.

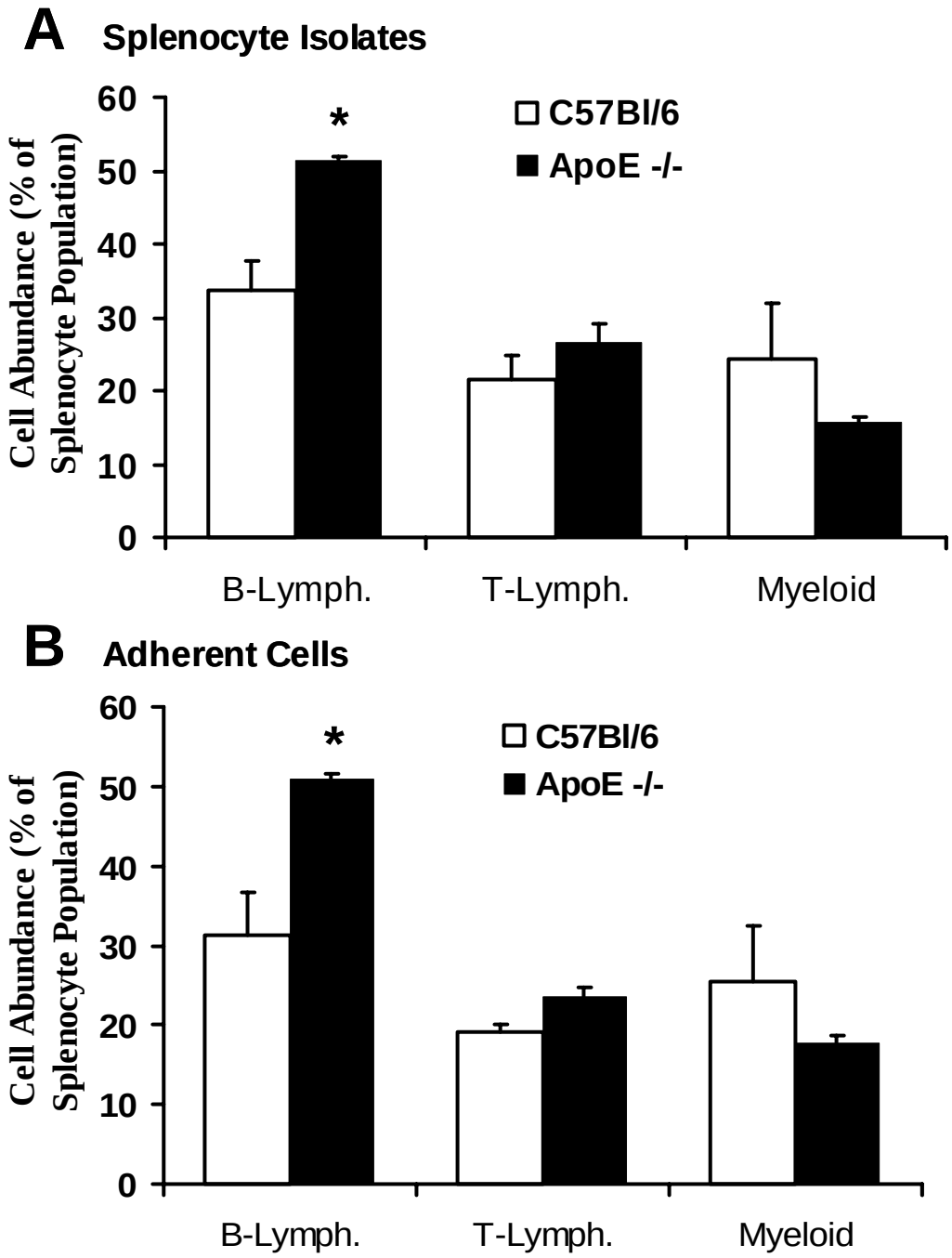
Figure 3: The effect of NCX 6550 and pravastatin administration on adhesion of isolated splenocytes to thrombin-treated artery segments from both wild type (panel A) and ApoE -/- (panel B) mice. Percent adhesion refers to the percentage of cells applied to the vessel segment that remained adherent after washing, which was determined as described for figure 2A. The results are presented as mean ± s.e.m. with n=11 for wild type and n=13 for ApoE -/- mice. *P<0.05 compared to vehicle-treated controls.

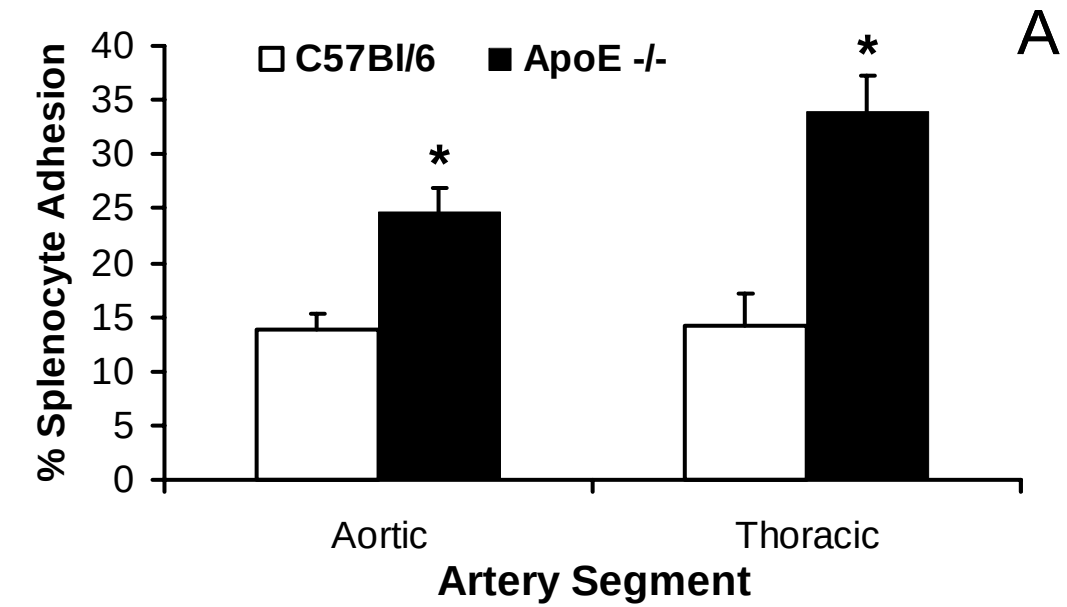
Figure 4: Immunocytochemical staining scores (arbitrary scale) for ICAM-1 in thrombin-treated arterial segments from C57Bl/6 and ApoE -/- mice treated with vehicle,

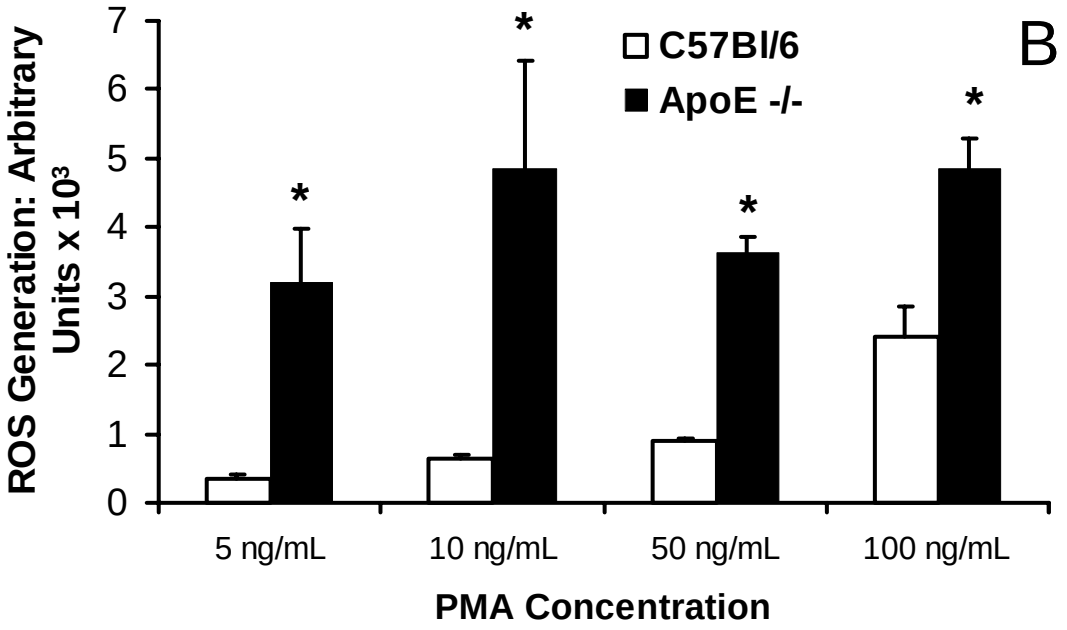
pravastatin or NCX 6550. *P<0.05; **P<0.01; ***P<0.001 compared to vehicle treated control mice (n=6 per group). Details of the scoring system are described in the Methods.

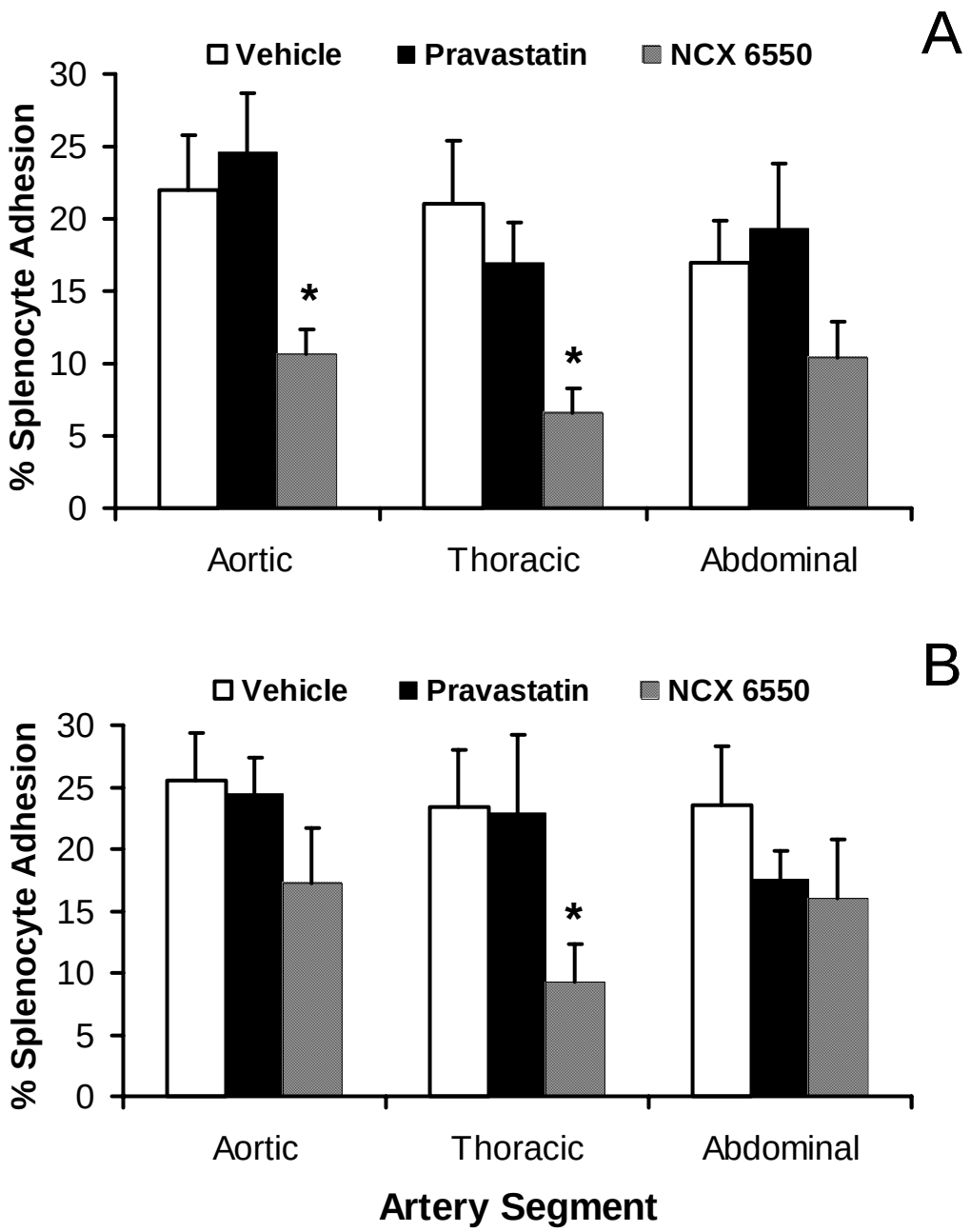
Figure 5: The effect of NCX 6550 and pravastatin administration on ROS production from C57Bl/6 splenocytes (panel A) and ApoE -/- splenocytes (panel B). ROS production was measured as described for figure 2B. Data are presented as mean ± s.e.m (n=4-6 per group); *P<0.05; **P<0.01 compared to vehicle control.

Figure 6: The effect of NCX 6550 and pravastatin administration on endothelial function in isolated aortic segments from ApoE -/- mice. Endothelial relaxant response was assessed by wire myography, with pre-constriction induced by 0.2 μM phenylephrine and relaxation by cumulative addition of carbachol. The results were analysed using Prism graph plotting software to calculate E_{max} and -logEC_{50}, and are shown as mean ± s.e.m (n=4). *P<0.05 (NCX vs pravastatin) ***P<0.001 (NCX vs vehicle) (Two-way ANOVA).









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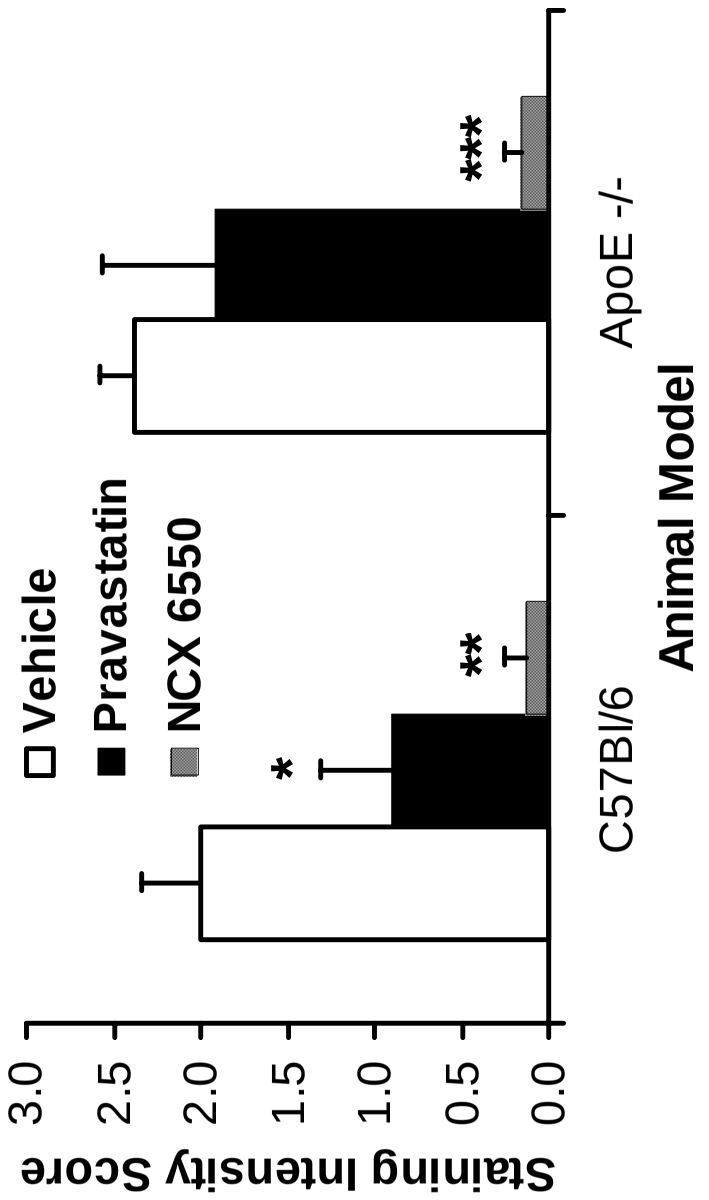

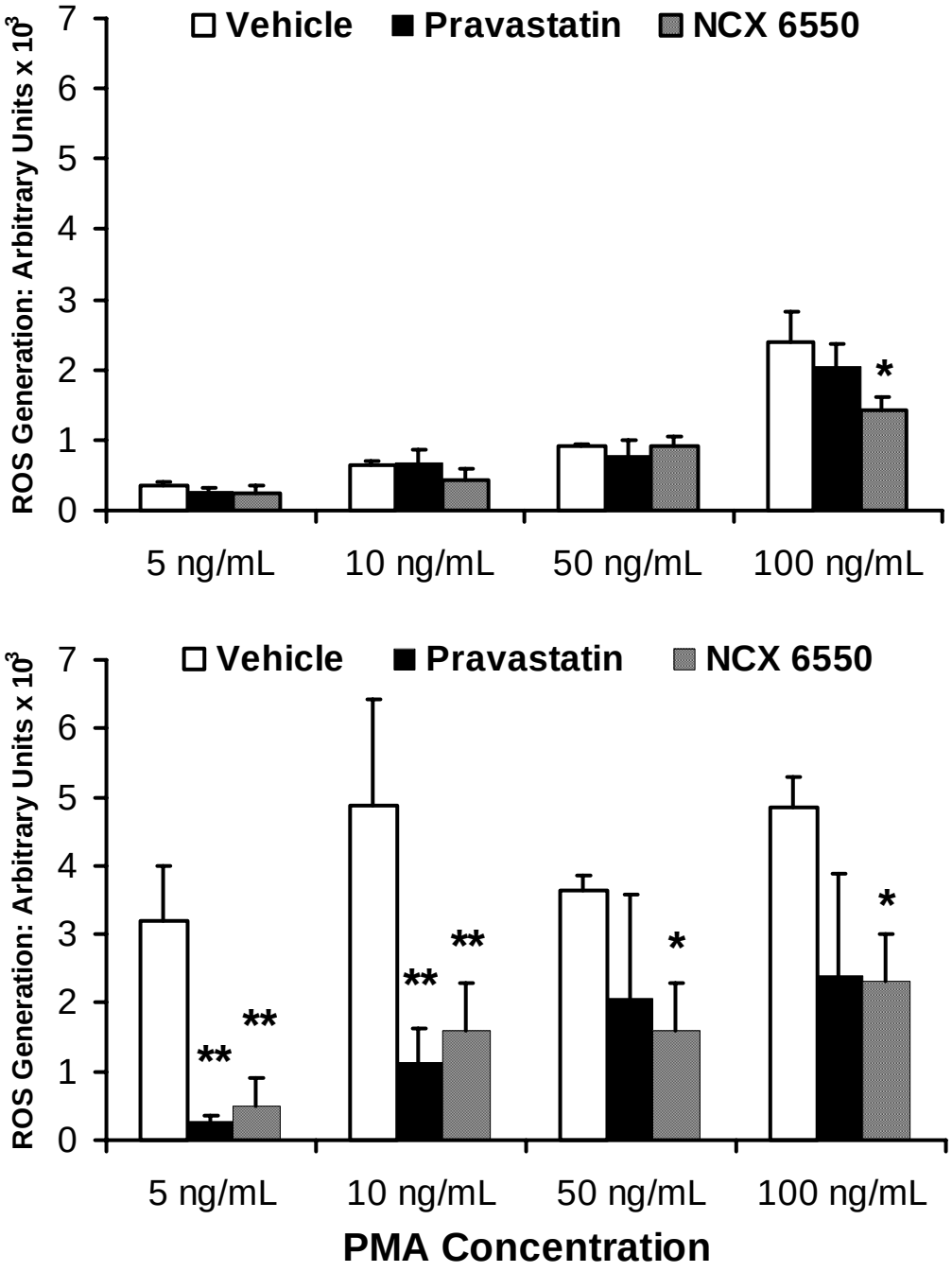

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